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TITLE: Nanotechnology-Based Detection of Novel microRNAs for Early Diagnosis of Prostate Cancer

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<b>14. ABSTRACT:</b> Prostate cancer (PCa) is most commonly diagnosed and second leading cause of death in the US men, representing 8% of all male cancer deaths. Currently, PCa is monitored and managed by prostate-specific antigen (PSA) screening combined with digital rectal examination (DRE). PSA screening had made a paradigm-shifting impact on the detection and management of PCa. However, these tests have limitations, because of low predictive value (25-37%) and high false positive results due to lack of sensitivity and specificity, resulting in significant "overtreatment of PCa patient. Hence, there is an urgent need to develop novel, non-invasive and more accurate biomarkers in conjugation with 'highly sensitive detection platform' for more precise diagnosis of PCa. Recently, an altered expression of miRNAs (miRs) have been observed in PCa and correlated them as a potential biomarker for PCa. In the present study, we are quantifying the expression level of deregulated miRNAs in mouse and human PCa tissues as well as serum samples using an advanced nanotechnology-based sensing nanoparticle probes (i.e. nanoprobe). Overall, the proposed studies will lead to the development of efficient technology for the accurate and early detection of differentially expressed miRNAs using sensitive Au-nanoprobes. This could enable us to develop early detection markers for PCa and differentiate between androgen independent and aggressive PCa. Recently, we have successfully synthesized and characterized fluorescently-labeled DNA-gold nanoparticle probes via bioconjugation. The result of fluorescence-based assay revealed that successful conjugation of ~150-200 oligonucleotides per Au nanoparticle. In parallel, our qPCR analysis on prostate tissues excised from PTEN conditional knockout and wild-type littermate controls identified differential expression level of miRNAs. We also analyzed expression patterns of miRNAs (identified through meta-analysis of previously published reports) in mouse PCa tissues and observed that significant overexpression of miR-21, miR-141 and miR-375 in PCa tissues compared to healthy tissue. In future, we will be quantifying an aberrantly expressed miRNAs level in mouse and human serum samples using our Au nanoprobe and their relevance for early detection and differentiation between androgen dependent and independent PCa.					
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## 1) Introduction

Prostate cancer (PCa) is major health problem worldwide and is the second leading cause of cancer-related deaths in the US men. ACS has reported 180,190 new cases of PCa have been diagnosed and around 26,120 estimated new deaths in 2016 [1, 2] and this accounts for 8 % of all cancer-related deaths among the US men [1]. Hence, an effort for early detection of PCa should be undertaken to improve the survival rate of patients. Currently, PCa is monitored and managed by prostate-specific antigen (PSA) screening combined with digital rectal examination (DRE). Serum PSA level more than 2.5-4 ng/mL and abnormalities observed during DRE may indicate the presence of PCa. The PSA is specific to the prostate in men but not to prostate malignancy, even in non-cancerous prostate disease conditions (i.e. patients with prostatic or benign prostatic hyperplasia (BPH)) can also have increased serum PSA level. Although serum PSA test along with DRE is a current gold standard for monitoring of PCa unfortunately, their positive predictive value is only 24-37 % respectively, resulting in a high rate of prostate biopsies and overtreatment. Hence, there is an urgent need to develop high sensitive detection platform to identify novel, more specific and sensitive, non-invasive PCa biomarkers to increase an accuracy of PCa diagnosis and to differentiate between androgen dependent (AD) to androgen independent (AI) PCa. Recent studies have shown that importance of circulating miRNAs (Cir-miRNAs)[3] as a promising biomarker because of their altered expression in various cancers including PCa [4, 5]. Further, differential expression patterns of miRNAs have also been reported in AD and AI PCa [6]. Thus, miRNAs could be an ideal candidate to study PCa pathogenesis, to identify early diagnosis markers and to differentiate between aggressive PCa to non-aggressive disease. However, sensible detection of miRNAs level in the body fluids (blood, urine etc.) tissue samples is remains difficult process, because of lack of selective and sensitive methods. Moreover, conventional methods (i.e. qRT-PCR, next generation sequencing, Northern blot, etc.) have shown some limitations i.e. weak or moderate sensitivity, less specificity, expensive instrumentation, extreme care to avoid contamination, etc. [7]. Therefore, there is an immediate necessity of nanotechnology-based innovative diagnostic platform to identify novel differentially expressed miRNAs in the body fluids (blood, urine, etc.) for an early detection of PCa. Advances in nanotechnology and availability of multiple types of nanomaterials have led to the development of sensitive assay platforms to the novel miRNA based markers for early diagnosis [8]. In recent years, research was directed to develop several nanoscale probes, alongside the discovery of the wide range of biomarkers to lower the detection limit of biomarkers. Therefore, in current proposal our goal is to establish a sensitive detection platform for detection of novel PCa biomarker (i.e. miRNA) for early diagnosis of PCa, distinguishing indolent vs. aggressive and hormone-dependent vs. independent PCa. Here, we have proposed the development of well-characterized gold nanoparticles (AuNPs)-based miRNA detection probes (i.e. Au-DNA nanoprobe) implemented for direct (without converting into cDNA) and sensitive detection of altered miRNA level during PCa progression, which can enable differentiation between aggressive and indolent cancer. Therefore, three different goals were laid out for the study. 1) Development of sensitive gold nanoprobe for direct and sensitive detection of miRNAs 2) Investigate stage specific global miRNA expression profiles in the PCa mouse tissue followed by their detection in mouse serum using nanoprobe. 3) To investigate the diagnostic potential of differentially expressed miRNAs identified in mouse model and their relevance in sera of human prostate cancer patients.

## 2) Keywords

Gold nanoparticles, DNA-Gold nanoprobe, microRNA quantification, Prostate cancer diagnosis, fluorescence assay.

## 3) Accomplishments

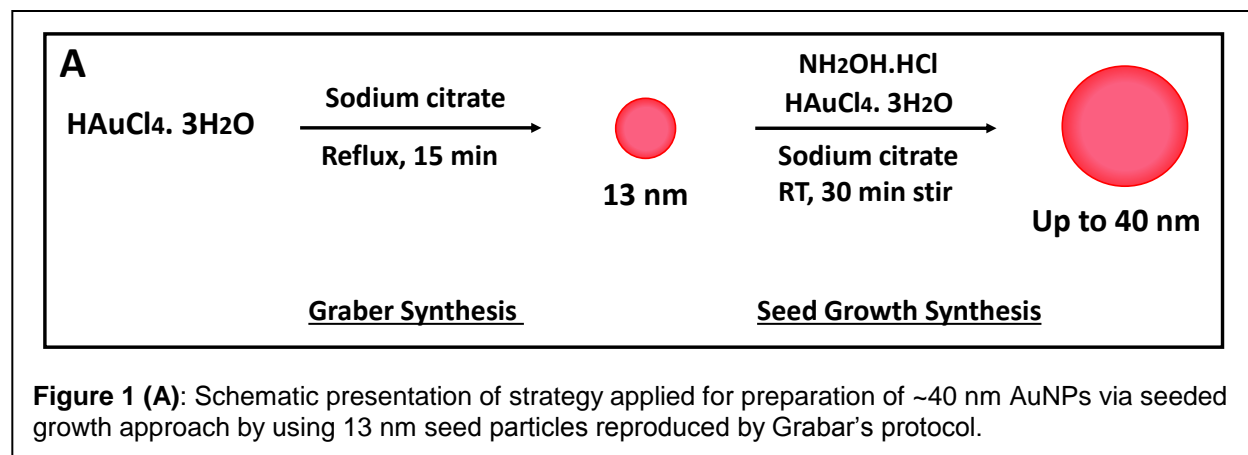
During the previous year, with the funding support from Department of Defense, we have performed the proposed experiments as per the approved statement of work (SOW) and have made following accomplishments.

**Major goals 1:** Development of fluorescent-tagged-oligonucleotide conjugated ~ 40 nm gold nanoparticle (DNA-AuNPs) based sensitive probes and their detailed characterization. (Completed- 40 nm gold nanoprobe preparation and characterizations part, as described in subtype 1 and 2)

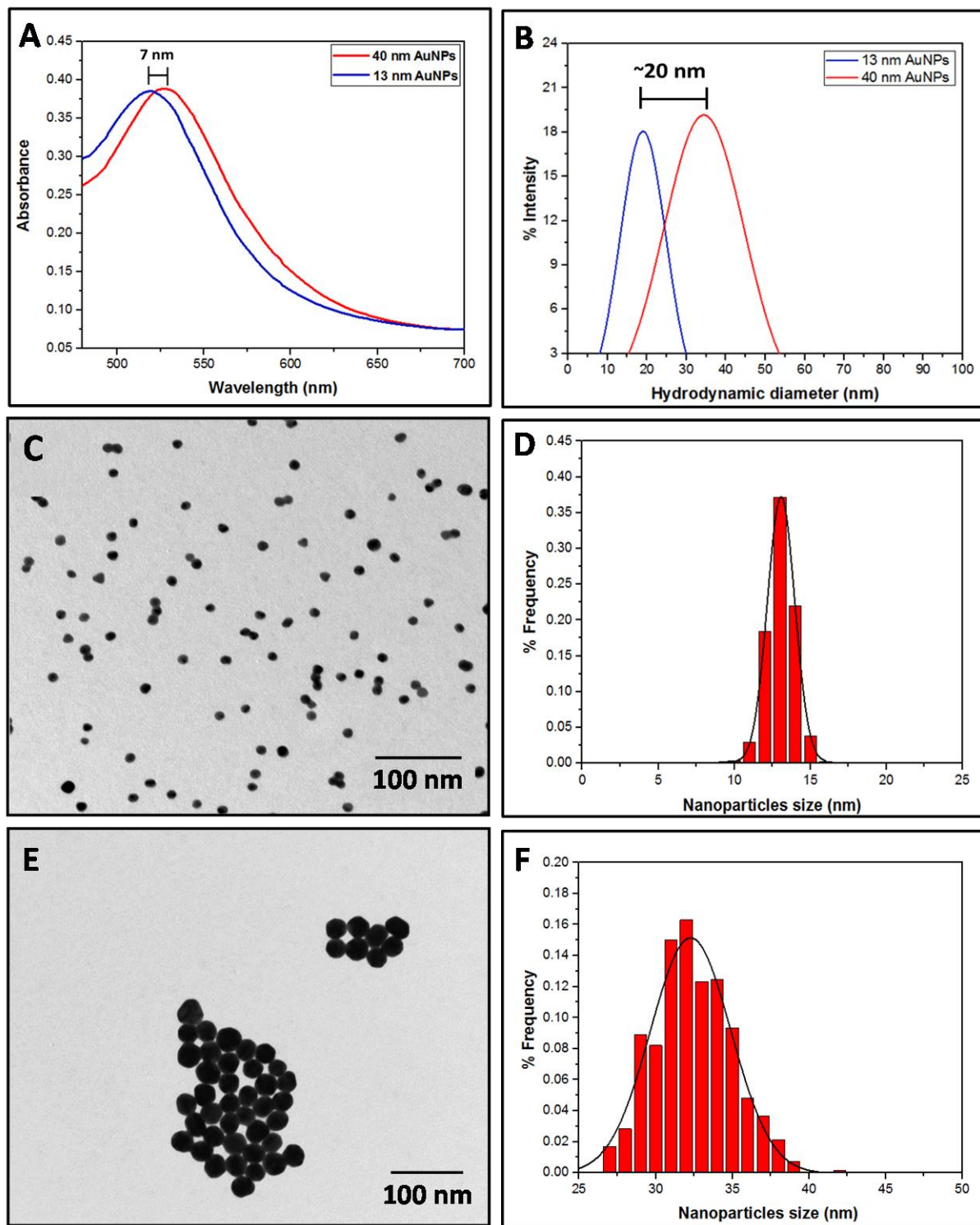
**Subtask-1:** Synthesis, detailed characterization of metal nanoparticles [i.e. Average ~ 40 nm gold nanoparticles (AuNPs)], PEG passivation of AuNPs

### a) Aqueous Synthesis of Gold Nanoparticles (AuNPs)

For making Au nanoprobe, we initiated with an aqueous synthesis of AuNPs (average size ~40 nm) and their detailed characterizations. First, we performed a synthesis of ~13 nm of Au colloids (AuNPs seed) described by Grabar et al. Further, well characterized Au seeds were used for making of 40 nm AuNPs via seeded growth approach (Fig. 1A, experimental scheme). In short, monodispersed aqueous 40 nm AuNPs was prepared following a kinetically controlled growth strategy via reduction of chloroauric acid ( $\text{HAuCl}_4$ ) by hydroxylamine hydrochloride ( $\text{NH}_2\text{OH}\cdot\text{HCl}$ ).



Further, we applied various techniques to performed characterizations of citrate stabilized/capped AuNPs in order to know the core sizes ( $\phi$ ), hydrodynamic diameters (HDs), shapes, zeta potential ( $\zeta$ ) (i.e. surface charge), polydispersity index (PDI) and localized surface plasmon resonance (LSPR) absorption. Following figure 2 is summarizing the analysis of the initial seed and seeded growth nanoparticles.

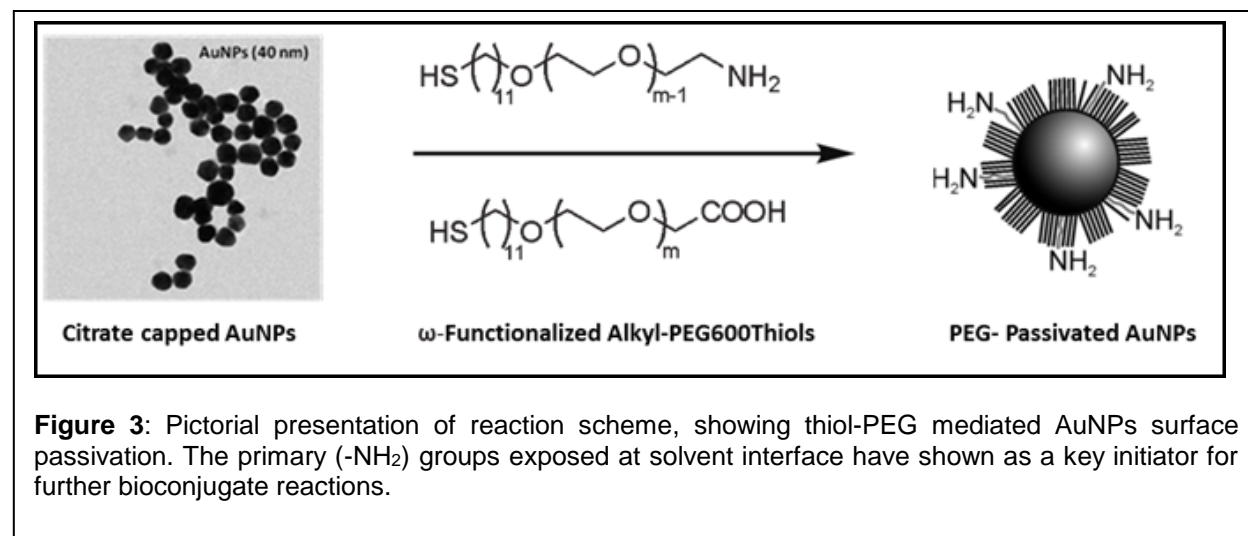


**Figure 2:** (A) UV-Vis spectrum of purified AuNPs i.e. 13 nm seed particles (Blue line,  $\lambda_{\max}$  520 nm) and 40 nm AuNPs (Red line,  $\lambda_{\max}$  527 nm) [Note: ~7 nm red shift in  $\lambda_{\max}$  value indicating an increment in the particle diameter]. (B) The DLS spectrum of purified AuNPs indicating ~20 nm shift in the hydrodynamic diameter (HD) suggestive of size growth of seed NPs. The average HD values for both seed and 40 nm particles are  $19.01 \pm 5.1$  nm (Blue curve) and  $39.04 \pm 10$  nm (Red curve) respectively. (C) Representative TEM image of AuNP seeds (Average size ( $\phi$ ) =  $13.01 \pm 1$  nm), and (D) Histogram (fitted with Gauss function) of seed NPs size distribution. (E) TEM image of AuNPs after seeded growth ( $\phi$  =  $33 \pm 2.5$  nm), calculated from size distribution histogram (F). Note: ImageJ and OriginLab 8.0 (2016) software were used to counting the size of individual nanoparticle (NP) and to draw the size distribution histogram.

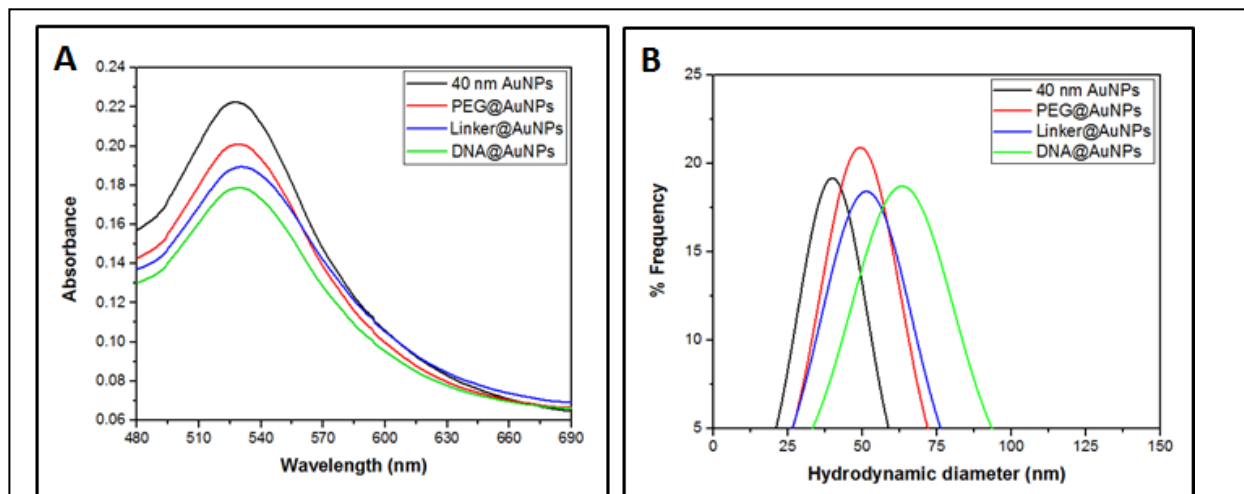
Detail interpretation of UV-Visible absorbance spectra (Fig. 2A) showed the LSPR absorption at 520 nm and 527.9 nm for 13 nm seed particles (Blue line) and 40 nm AuNPs (red line) respectively. UV-Visible spectral analysis have indicated the red shift of  $\sim 7.0$  nm in absorption maximum ( $\lambda_{\text{max}}$ ) as AuNPs size grown from 13 nm to 40 nm. Parallely, we also have observed changes in hydrodynamic diameters (HDs) of the both synthesized seed (blue curve) and 40 nm AuNPs (red line) via dynamic light scattering (DLS) measurement (Fig. 2B). The average HDs were observed to be  $19.01 \pm 5.1$  nm (PDI = 0.11) and  $39.04 \pm 10$  nm (PDI = 0.09 - 0.11 range) respectively. The DLS spectrum (Fig. 2B) clearly indicating the increment in the HDs of  $\sim 20$  nm, suggesting growth of seed particles to the higher size diameter. However, an actual core size and dispersity of NPs was validated by performing transmission electron microscopy (TEM) imaging. Representative TEM pictures (Fig. 2C and 2E) have shown monodispersed and uniform size [i.e. average circularity ( $\sim 0.89$ ) and ellipticity ( $\sim 1.10$ )] and spherical in shape. The average value of AuNPs diameter ( $\phi$ ) were obtained by using ImageJ software. Size distribution histogram (fitted with Gaussian function) of AuNPs were obtained by analyzing more than 300-800 NPs. After analyzing TEM pictures with ImageJ software, we observed the average core size of the seed NPs ( $\phi = 13.01 \pm 1$  nm) and seeded growth AuNPs ( $\phi = 33 \pm 2.5$  nm). The zeta potential ( $\zeta$ ) analysis of both citrate-capped particles shown negative surface charge (Average  $\zeta$ -value mentioned in Table-1) Furthermore, these well characterized 40 nm size, spherical shaped AuNPs were used for making of Au nanoprobe via surface modification (i.e. PEG passivation, Linker binding and fluorescence-tagged oligonucleotide conjugation).

## b) PEG passivation

The passivation of AuNPs (i.e. formation of mixed monolayer ligand shell on NP core) were performed by treating the AuNPs with mixed solution of amine- and carboxyl-terminated poly(ethyleneglycol)-based thiols (PEG-SH). After 48 hours of incubation at room temperature, uniform and stable monolayer of PEG-SH were formed on AuNPs surface. The primary amine ( $-\text{NH}_2$ ) headgroups at the solvent-expose interface were used for further functionalization of particle surface with active biomolecules via connecting linker. We performed AuNPs passivation by varying the mole fraction of amine-terminated PEG-SH (0.1 - 0.33  $\mu\text{M}$ ) to tune the number of surface biomolecules later in the DNA conjugation step (Table 2). Again, we performed NP analysis using various characterization techniques as mentioned above.



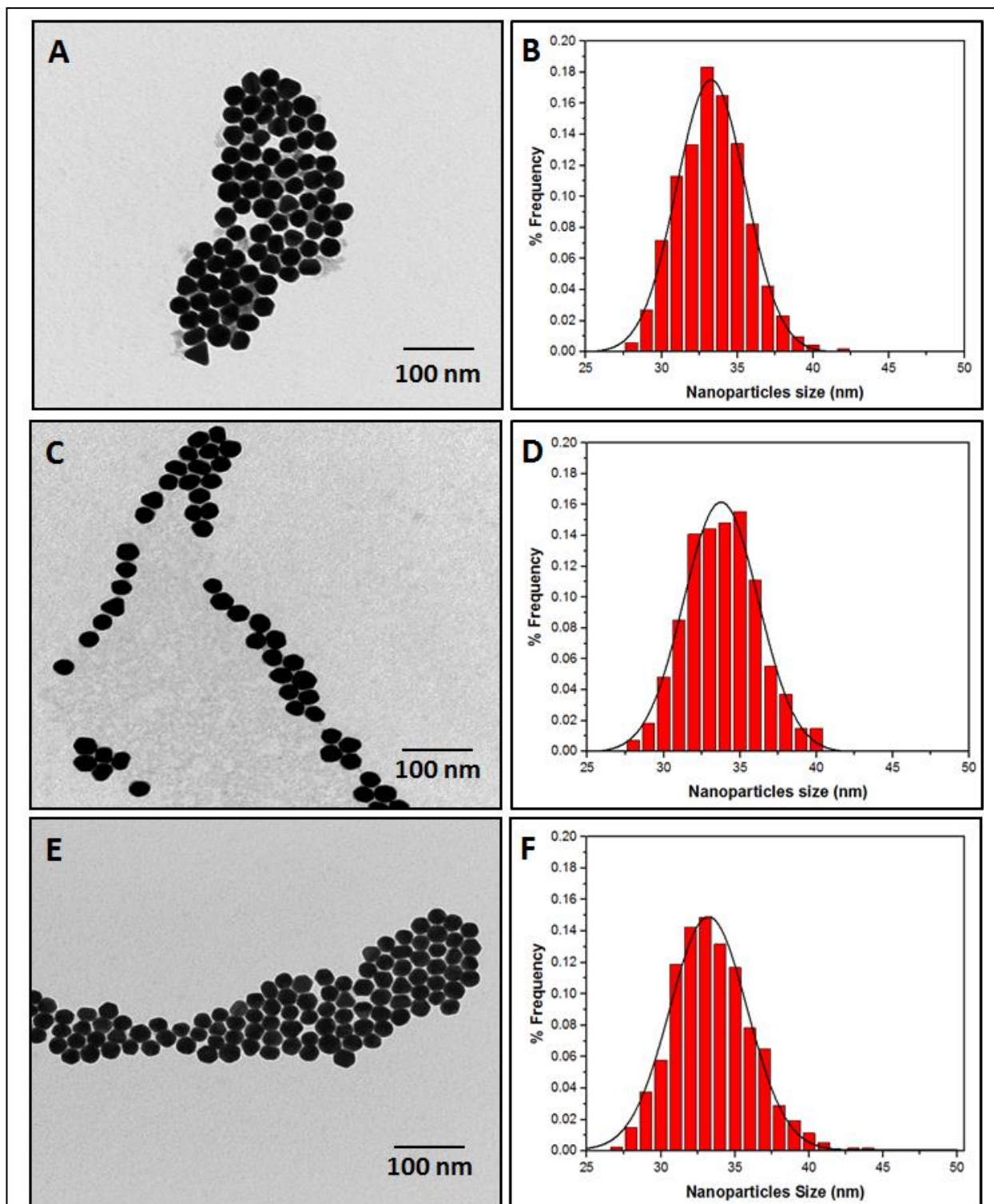
UV-Vis analysis (Fig. 4A, red line) have shown SPR absorption ( $\lambda_{\text{max}}$ ) at 529 nm while the DLS size histogram (Fig. 4B, red line) representing an increment in the average HD value (HD =  $49.3 \pm 13$  nm, PDI = 0.12), showing successful PEG-SH (Final concentration of 0.25  $\mu\text{M}$  in reaction) passivation of AuNPs (PEG@AuNPs). The HD value was verified by nanoparticle tracking analysis (NTA) technique (NanoSight) which closely matching with (HD=  $51.9 \pm 11$  nm). The observed zeta ( $\zeta$ ) value for PEG@AuNPs was  $-41.0 \pm 3.0$  nm, indicative of negatively charged surface carboxylate ( $-\text{COO}^-$ ) group. Additionally, TEM imaging of PEG@AuNPs (Fig. 5A), showing AuNPs are approximately spherical in shape and reasonably monodispersed (distinctly separated) compared to the bare AuNPs, indicating the successful surface covering or coating of PEG-thiol molecules. TEM image (Fig. 5A) and size histogram (Fig. 5B) analysis were also showed that majority of NPs are spherical in shape, while few of them have displayed somewhat faceted shaped, as a result of their nanocrystalline nature. The average size of PEG@AuNPs was calculated using ImageJ software, and black curve (Fig. 5B) after Gauss fitting showed average core size of NPs was  $33.3 \pm 2.3$  nm. Furthermore, TEM pictures and calculated core size has also signified the no significant difference between core size of bare and PEG-coated AuNPs. That would observe, as only the gold core is visible at the accelerating voltage used in TEM instrument. Throughout the all the reactions, passivated AuNPs was found stable and very resistance to irreversible aggregation. In short, the primary purpose behind passivation is to maintain good water solubility, enhanced the stability toward aggregation and insist a base for further surface modification. Currently, we are standardizing a detection strategies for miRNAs (isolated from PCa mouse tissue and from serum samples) using 40 nm Au-nanoprobe. We will also use different sizes (lower and higher sizes) in order to enhanced detection sensitivity i.e. lowering the limit of detection (LoD).



**Figure 4:** Pictorial presentation of reaction scheme, showing thiol-PEG mediated AuNPs surface passivation. The primary ( $-\text{NH}_2$ ) groups exposed at solvent interface have shown as a key initiator for further bioconjugate reactions.

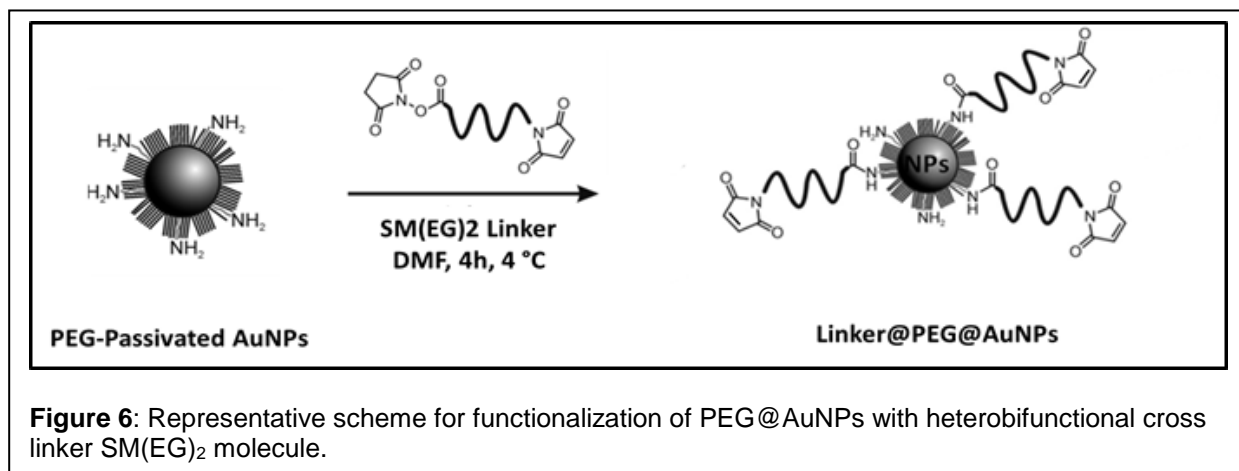
## **Subtask 2:** Synthesis and characterization of DNA-AuNP Nanoprobe

### **a) Surface functionalization of PEG@AuNPs with SM(EG)<sub>2</sub> Linker:**



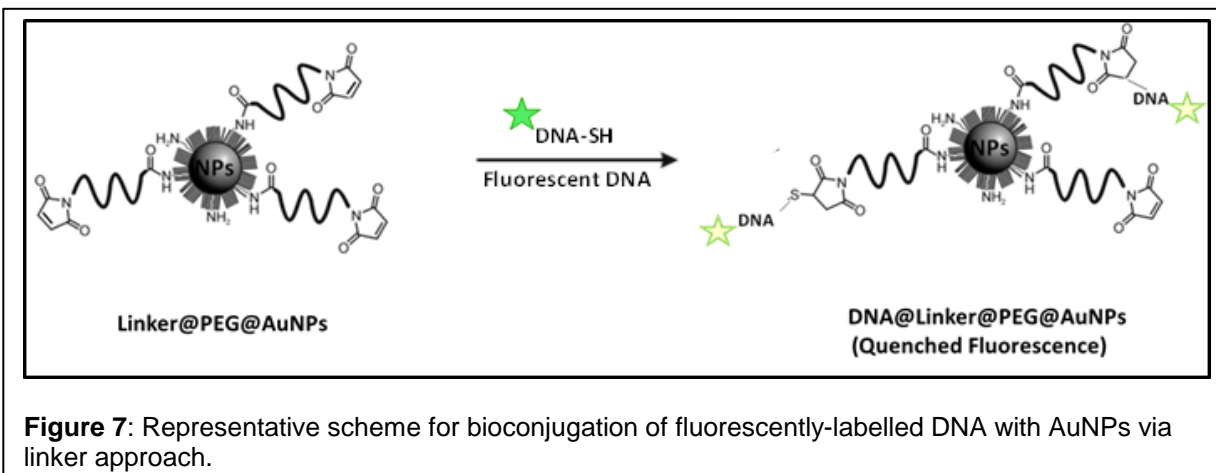
**Figure 5:** Pictorial representation of TEM images of AuNPs (Left panel) and respective size distribution histogram (Right panel) fitted with Gaussian curve (black line). Image **A** and histogram **B** of PEG@AuNPs, with average size ( $\phi$ ) =  $33.3 \pm 2.3$  nm core size calculated from histogram. Image **C** and histogram **D** of linker@AuNPs, ( $\phi$ ) =  $33.7 \pm 2.45$  nm core size, Image **E** and histogram **F** of DNA@AuNPs with ( $\phi$ ) =  $33.2 \pm 2.6$  nm core size. Note: ImageJ and OriginLab 8.0 (2016) software were used to counting the size of individual nanoparticle (NP) and to draw the size distribution histogram.

PEG passivated AuNPs was surface functionalized with hetero-bifunctional cross linker succinimidyl-[(N-maleimidopropionamido)-diethyleneglycol] ester [i.e. SM(EG)<sub>2</sub>]. In short, a few hours incubation of aqueous PEG@AuNPs with (SM(EG)<sub>2</sub> linker molecules (dissolved in DMF solvent) and successive purification yielded linker-bound Au particles (i.e. Linker@AuNPs). Here, the more labile NHS-Ester functionality was reacted with exposed –NH<sub>2</sub> group at the surface of AuNPs (Figure 6, Linker-binding reaction scheme). Additionally, we have also performed a control experiment, where an aliquot of PEG@AuNPs was treated with equal amount of DMF solvent, omitting the SM(EG)<sub>2</sub> linker.



The UV-Vis and DLS characterization of linker-functionalized AuNPs (Linker@AuNPs) showed SPR maxima peak ( $\lambda_{\text{max}}$ ) at 529 nm (Fig. 4A, blue curve), and DLS spectra (Fig. 4B, blue curve) displayed HD value  $51.3 \pm 15$  nm (PDI = 0.13). The unchanged  $\lambda_{\text{max}}$  value compare to bare and PEG-coated NPs indicating core size that the actual core size is unchanged. However an increment in the HD value compare to bare AuNPs revealed surface modification of AuNP surface. TEM analysis shown that NPs are well dispersed and stable (Fig. 5B) with the average core size ( $\phi$ ) of  $33.7 \pm 2.45$  nm (Fig. 5C). The purpose of linker binding is again to make AuNPs more stable, water soluble and set up a base for the next step of oligonucleotide conjugation.

#### **b) Conjugation of 6-carboxyfluorescein (FAM) labeled probe oligonucleotide to linker functionalized AuNPs (Linker@AuNPs) and purification:**



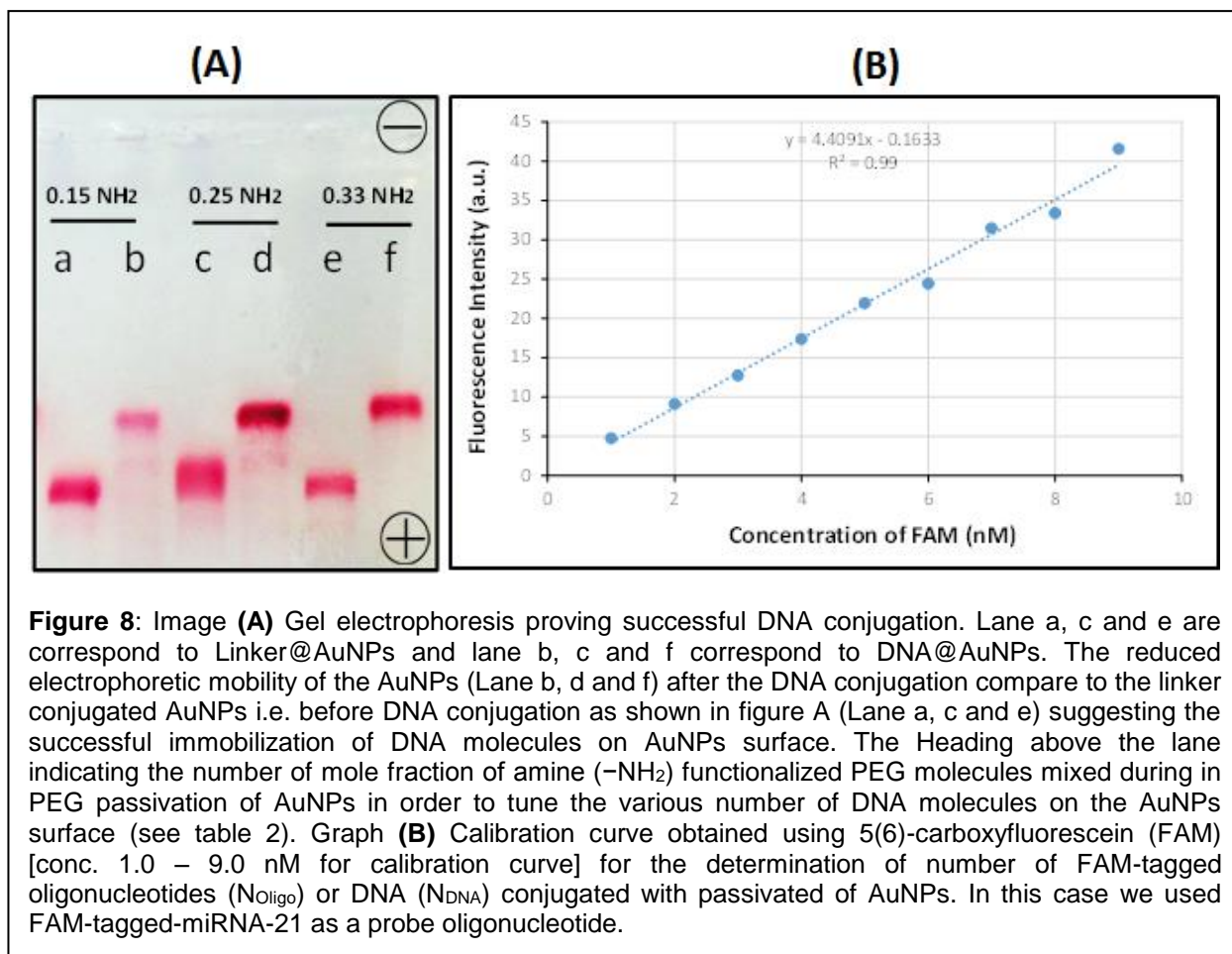
In short, a 3'-thiol group of the probe oligonucleotide (i.e. DNA-probe) sequence specific for detection miRNA-21(DNA probe) was deprotected and purified through gel filtration. Later, freshly deprotected miRNA-21 sequence was incubated directly with linker@AuNPs to form 'oligonucleotide-AuNPs (i.e. DNA-AuNPs)' conjugates (Fig. 7, bioconjugation scheme) and was further purified with gel filtration using NAP-5 column. Further, we perform surface analysis to check the NP size, shape, stability of conjugates and gel electrophoresis to confirmed oligonucleotide (i.e. DNA) and AuNPs bioconjugation. The SPR absorption ( $\lambda_{max}$ ) for DNA-AuNPs was observed at 529 nm (Fig. 4A, green curve), however DLS curve (Fig. 4B, green curve) representing HD size ( $HD = 63.2 \pm 17.1$  nm,  $PDI = 0.16$ ). In summary, Figure 4A and 4B demonstrated that, the HD value was incrementally increases compare to bare, PEG- or linker-functionalized AuNPs. This suggesting a dense coverage of probe DNA (i.e. FAM-miRNA-21 Probe) over the passivate shell of AuNPs without affecting the core sizes of all AuNPs (see Fig. 5 A-E). TEM picture and correlated histogram (Fig. 5 E and F) have also shown average size of  $33.2 \pm 2.6$  nm. The Zeta ( $\zeta$ ) value was recorded to be  $-35 \pm 3.1$  nm, telling that DNA@AuNPs are negatively charged particle and do indeed migrate towards the positive electrode in electrophoresis experiment (Fig. 8A). Here, we are summarizing the out comings from the first part of our major goal (i.e. subtask 1 and 2).

#	AuNPs type	Size (DLS, nm)	PDI (DLS)	Size (NTA, nm)	$\zeta$ -potential (NTA, mV)	Size (TEM, nm)
1	13 nm AuNPs (Seed NPs)	$19.01 \pm 5.0$	0.14	NA	NA	$13.1 \pm 1.00$
2	40 nm AuNPs (Seed growth NPs)	$39.04 \pm 10$	0.09	$39.04 \pm 10$	$-37.2 \pm 2.9$	$33.0 \pm 2.51$
3	PEG@AuNPs	$49.3 \pm 13$	0.12	$45.3 \pm 13$	$-41.1 \pm 3.0$	$33.3 \pm 2.30$
4	Linker@AuNPs	$51.3 \pm 15$	0.14	$55.3 \pm 15$	$-34.9 \pm 3.2$	$33.7 \pm 2.45$
5	DNA@AuNPs	$63.2 \pm 17$	0.17	$61.2 \pm 17$	$-35.1 \pm 3.1$	$33.2 \pm 2.60$

**Table 1:** Summary of major goal 1 (i.e. subtype 2a and 2b) mentioning the characterization of various types of AuNPs (as stated in column 2) via UV-visible, DLS, NTA and TEM analysis.

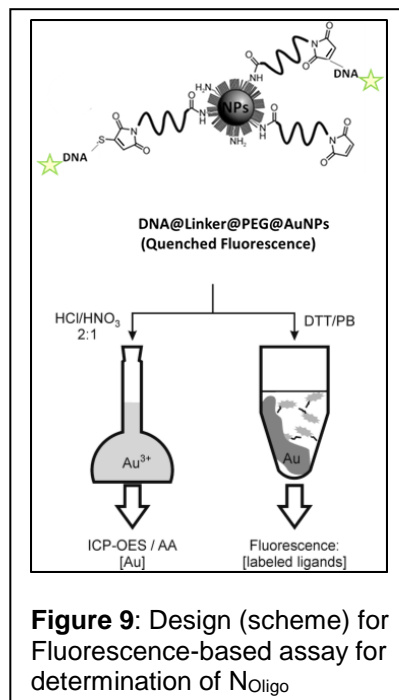
### C) Characterization of DNA@AuNPs (Nanoprobes) via Gel electrophoresis:

A purified and well characterized nanoprobes (Table 1) were characterized by gel electrophoresis in order to confirm the successful conjugation of fluorescently-tagged DNA molecules on passivated layer of AuNPs. After bioconjugation reaction, both a linker@AuNPs and DNA@AuNPs were analyzed on agarose gel to check the electrophoretic mobility. We observed a band with a clearly reduced electrophoretic mobility for DNA@AuNPs probes (Fig. 7, lane 2) compare to the linker@AuNPs particles (Fig 7, lane 1). For bioconjugation experiment, we have used three different types of PEG-passivated AuNPs (as mentioned earlier) by varying mole fraction of amine-terminated PEG-SH (0.1 - 0.33  $\mu$ M). Hence, we have three different probes with varying the number of fluorescently-tagged DNA molecules on AuNP surface (Table 2).



#### D) Quantification of the number of coupled oligonucleotides per AuNP (N<sub>Oligo</sub>):

To quantify the FAM-tagged oligonucleotides per AuNP, we performed two parallel experiments (Fig. 9, Scheme) to know the concentration of fluorophore (i.e. FAM) and concentration of DNA@AuNPs. Two aliquots of DNA-AuNPs were withdrawn, the first one was treated with Dithiothreitol (DTT)-Phosphate buffer solution and performed fluorescence assay in order to determine the concentration of coupled FAM-tagged oligonucleotides. We calculated AuNP-conjugated oligonucleotide's concentration by comparing the resulting fluorescence intensity (from DTT experiment) with respective FAM concentration (in nM) shown in calibration curve (Graph 8B). Another aliquot for determine concentration via Induced Coupled Plasma- optical emission spectrometry (ICP-OES) technique. *Finally, number of FAM coupled oligonucleotide was calculated above formula: (N<sub>Oligo</sub> = [Conc. of FAM] / [Conc. of AuNPs]).*



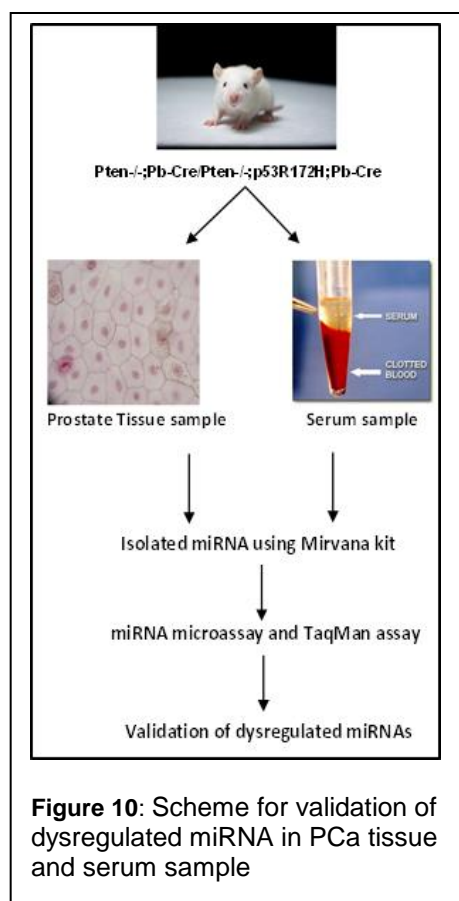
Following table representing the short summary of the subtask 2C and 2D.

#	AuNPs type	Mole fraction ( $\chi$ ) of HS-PEG-NH <sub>2</sub>	[Conc. FAM] nM	[Conc. AuNPs] nM	N <sub>Oligo</sub>
1	DNA@AuNPs	0.15	522.02	2.98	175
2	DNA@AuNPs	0.25	1135.2	7.45	152
3	DNA@AuNPs	0.33	989.92	4.77	207

**Table 2:** Summary of major goal (i.e. subtype 2C and 2D) mentioning number of oligonucleotide (N<sub>Oligo</sub>) per AuNP. Different type AuNPs were prepared by varying the mole fraction of Thio-PEG-NH<sub>2</sub> for AuNPs passivation.

**Major Goal 2:** To investigate stage specific global miRNA expression profiles in the PCa mouse tissue followed by their detection in mouse serum by using nanoprobe. (Completed: Validation of differential expressed of miRNAs in genetically engineered PCa mice (with/without castration) tissue sample.

**Subtask-1:** Determine the altered global expression profiles of miRNAs during PCa progression in mice having PCa with/without castration: Briefly, Dr. Batra's lab (Project mentor) has already generated *Pten<sup>fl/fl</sup>;Pb-Cre<sup>+</sup>;p53R172H* mice that spontaneously develops PCa. In our observation, we found that mice of 14-16 weeks of age started developing tumor in prostate. Hence, after genotyping, we divided mouse into two groups i.e. first group consisting of mice shown positivity for the lox p sites in the PTEN gene, mutated R172H, Rosa26 and probasin-Cre and 2nd groups with only wild type PTEN. We euthanized three mice in each group of 16 weeks of age (postnatal). Malignant and normal prostate tissues were excised and made into two parts. A part of the prostate was flash-frozen in liquid nitrogen for RNA isolation and the other part is fixed in buffered formalin for histological and pathological analysis. Furthermore, miRNAs/total RNAs (T-RNAs) were isolated from flash frozen PCa and normal tissue sample using MirVana RNA isolation kit (Thermo Scientific). We performed TaqMan assays (Applied Biosystems) using probes specific for miR-21, miR-141, miR-375, miR-210, miR-221, miR-155 and let-7b in those isolated miRNAs. These miRNAs were selected based upon their significant association in human PCa.

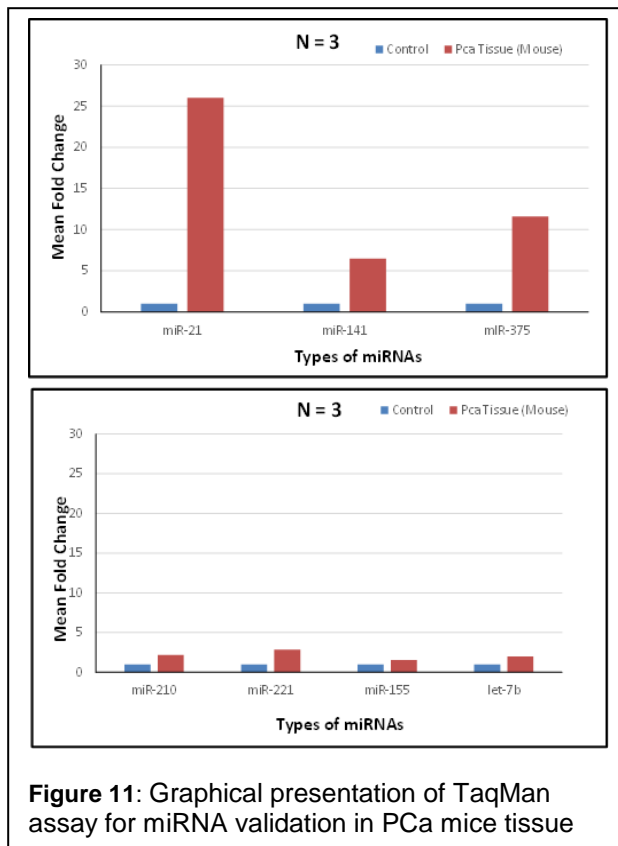


**a) TaqMan assay:** We performed this analysis using a standard TaqMan-assay kit (Applied Biology) and followed their standard protocol to perform study expression level of miRNAs as mentioned below. Importantly, the mean fold change in the expression level of particular miRNA will be calculated by using delta delta CT method. U6 snRNA was used as an internal control for normalization purpose. After meta-analysis of the literature report on miRNA in prostate cancer, we found that some the common miRNAs (i.e. miR-21, 141, 375, 210, 221, 155 and let-7b) with aberrant expression in PCa tissue, serum as well as tissue samples. Therefore, we selected the same miRNA for our study. Our experimental outcomes showed that miR-21, 141 and 375 are significantly upregulated in the PCa tissue excised from the p-ten conditional knockout mice harboring p53 mutation, compared to their littermate and internal controls while miR-210, 221, 155, and let-7b have shown only incremental change in expression in prostate cancer tissues compared to controls.

Since, it is first time we are implementing our nanoprobe on miRNAs isolated from genetically engineered PCa mice, various standardization has to be needed. Meanwhile, our next goal is to study and analyze the expression level of miRNAs in the serum (Sera) samples of genetically engineered PCa mice, and verified by sensitive Au-nanoprobe. In summary, we are establishing a nanotechnology-based sensitive probe for direct detection and quantification of deregulated or aberrant level of biomarker miRNAs in PCa for achieving early detection and differentiation of Ad and AI PCa

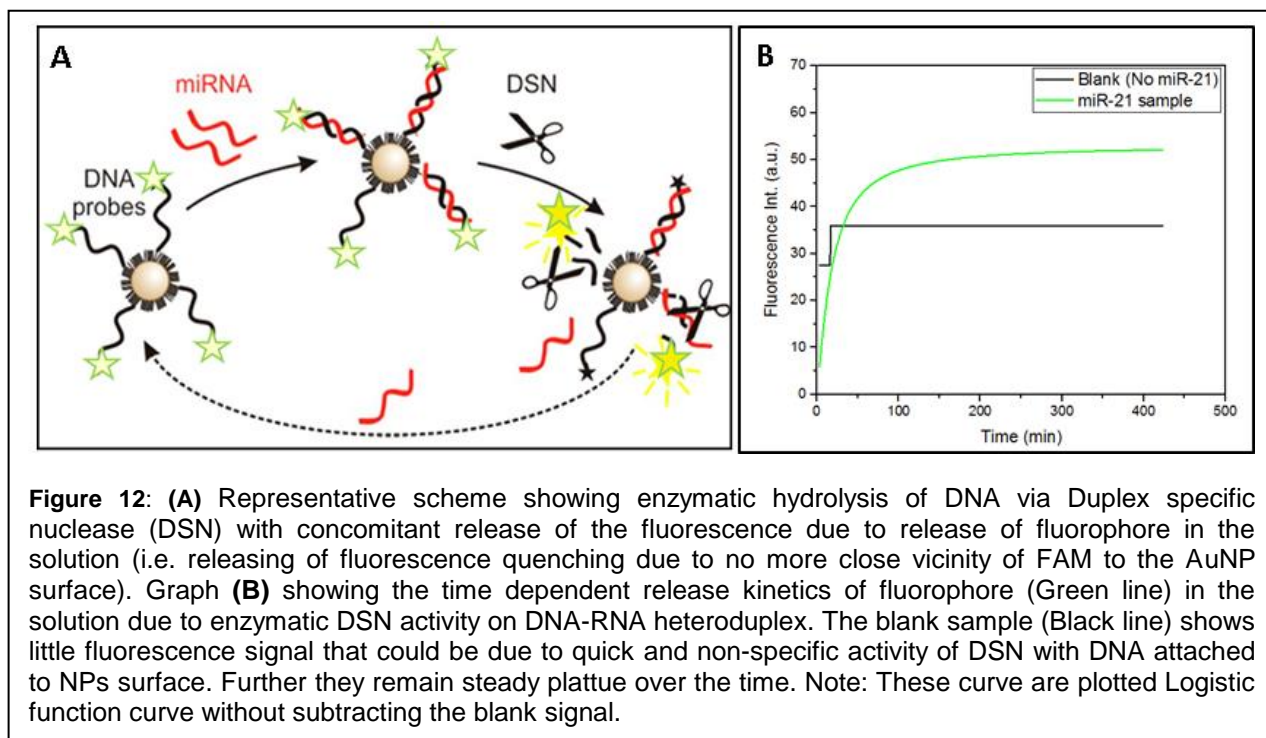
## b) Typical miRNA quantification assay

In case of our direct (without any cDNA conversion) miRNA quantification assay, typically DNA@AuNPs probes are implemented in a simple strategy for direct microRNA (miRNA) quantification. Fluorescently labeled DNA-probes (i.e. FAM-miRNA-21 probe) strands are immobilized on PEGylated gold nanoparticles (AuNPs). In the presence of target miRNA, DNA-RNA heteroduplexes are formed and become substrate for the endonuclease DSN (duplexspecific nuclease). Enzymatic hydrolysis of the DNA strands yields a fluorescence signal (Fig. 12B) due to diffusion of the fluorophores away from the gold surface (Figure 12 A). Recently we performed a pilot experiment to check the fluorescence release kinetics when total RNA isolated from PCa mice tissue used for detection (i.e. miRNA source).



**Figure 11:** Graphical presentation of TaqMan assay for miRNA validation in PCa mice tissue

As we mentioned earlier, we are in process to standardization of our assay with actual PCa samples. Here are the results from pilot experiment.



### Opportunities for Training and professional development provided by project

The PCRC-14 project has provided several training opportunities to work with new innovative techniques, ideas and suggestions from research discussion through journal clubs, seminars, and instrumental trainings public meetings. Being as a faculty member of BMB department, I myself got exposed to an excellent PCa training environment. My parent BMB department and Epplery Institute for Research in Cancer are continuously sponsoring a weekly seminar series by inviting an expertise to discuss and shares various research ideas, advance finding in cancer including PCa. I got a chance to develop my presentation skills by participating in monthly PCa Journal Club and departmental seminars in the BMB department. In addition, at our department PCa Research focus group, UNMC and chief of urological surgery conducts monthly meeting to discuss advances and shares their knowledge from the ongoing PCa research. I have also present my research outcomes in these meetings to get feedback, suggestions and also to develop collaborative and translational research with other basic scientists and clinicians. UNMC Postdoctoral Office conducts periodical workshops, including public speaking, grant writing, scientific writing and seminars on teaching skill and new techniques, where I personally learn a lot of basics. Individual monthly meeting with mentor (Dr. Batra) and co-mentor (Dr. Mahato), quarterly group meeting/work presentation serve as vital tools for input, guidance, suggestions, and solution for experimental problems from other researcher fellows. During the tenure of my training, I was also exposed to instrumental techniques, hands on molecular and

Cell biology, biochemical lab techniques, training of transmission electron microscopy (TEM), ICP-OES techniques. I have also attended UNMC SPORE retreat meeting at Nebraska city (1<sup>st</sup> June – 3<sup>rd</sup> June) and presented a seminar entitled “Direct and Absolute Quantification of micro-RNA (miRNA) using AuNP-\*DNA (Off-On) Probe” I am also planning to submit an abstract for incoming the 14th International Nanomedicine & Drug Delivery Symposium (nanoDDS’16) will be held at The Johns Hopkins University, Baltimore, Maryland, from September 16-18, 2016.

#### **Where the results disseminated to community of interest?**

“Nothing to report”

#### **Plan for next reporting period to accomplish the goal**

**Plan-1:** Successful development of DNA-nanoparticle based nanoprobe and set-up miRNA quantification protocol for mouse model: Based on the current outcomes, very first and basic thing we have planned to establish the standardized and reproducible miRNA detection assays for biological sample (i.e. mainly serum and tissue) using our well characterized Au nanoprobe.

**Plan-2:** To investigate the diagnostic potential of differentially expressed miRNAs identified in mouse sera sample and their relevance in sera of human prostate cancer patients. To do so our strategy will be identification of aberrant expression of miRNA isolated from human tissues as well as serum and the deregulated miRNAs (androgen dependent and independent prostate cancer) will be identified by the DSN mediated miRNA quantification assay using DNA-AuNPs Nanoprobe and miRNA microarray.

**Plan-3:** Successful development diagnostic nanoprobe for direct serum based assay for PCa patient. We are also planning to establish an advanced multiplexing detection approach for detection of multiple miRNA in serum sample. Detailed description of our plan was mentioned in the statement of purpose (SOP) document file.

#### **4) Impact:**

##### **Impact on the development of the principle discipline of the project**

The proposed studies in PCRP-FY2104 postdoctoral fellowship application will help to launch innovative nanotechnology based sensitive nanoprobe as a diagnostic tools for early detection of aberrant expression of miRNAs as a potential biomarker in various types of malignancy. Furthermore, our studies will investigate stage specific global miRNA expression profiles in the PCa tissue during murine PCa progression (Pten; p53; Pb-Cre model readily available in mentor’s lab) followed by their detection in mouse serum and in sera of human prostate cancer patients by using innovated nanoprobe. On the basis of current outcomes, we are confident that, the current proposal and our future studies will give results into the development of effective strategies for early detection of novel oncogenic miRNAs using sensitive Au based nanoprobe. Subsequently results into an effective diagnostic tools for PCa diagnosis as well as differentiate between androgen dependent to androgen independent PCa in patient. Overall the project will lead to the discovery of novel circulating biomarkers and development of novel detection technologies which in combination will be useful in

distinguishing indolent vs aggressive and hormone dependent vs independent PCa. Hence our proposal addresses and aligns with the PCRP-FY2014 focus area “Development of Biomarkers for PCa and early diagnosis”.

### **Impact on other disciplines and technology transfer**

Nothing to report. (Currently our project is under development, so could not really relates our current outcomes with other disciplines. However, there are some hopes of technology transfer of our model project in the near future.

### **Impact on society beyond science and technology**

Prostate cancer (PCa) is the most commonly diagnosed and second leading cause of cancer related mortality in US men. Lack of sensitive and specific technology resulting into the high incidence of patient death worldwide. In future, all these advanced nanotechnology-based research strategies will make a huge impact on early stage diagnosed PCa patients by providing them early stage medication/therapies and by contributing to the goal of eliminating death risk. It will be one step close towards the eradication of PCa from the society.

### **5) Changes/Problems**

Nothing to report

### **6) Product**

- 1) Review article: Currently we are in the process of writing a review article entitled- “Advanced Nanotechnology based Detection of Cancer Biomarkers”
- 2) Presentation: Presented in UNMC-SPORE retreat held at Nebraska city during (1<sup>st</sup> June – 3<sup>rd</sup> June 2016), seminar entitled “Direct and Absolute Quantification of micro-RNA (miRNA) using AuNP-\*DNA (Off-On) Probe”
- 3) Attending incoming the 14th International Nanomedicine & Drug Delivery Symposium (nanoDDS’16) will be held at The Johns Hopkins University, Baltimore, Maryland, from September 16-18, 2016.

### **7) Participant and Other Collaborating Organization**

Nothing to report

### **8) Appendices**

Nothing to report

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